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IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: Tadashi MIYASAKA et al

Serial No.: 627,980 Art Unit: 122

Filing date: July 5, 1984 Examiner: Gibson #6

Title of the Invention:

New Camptothecin Derivatives and Process for preparing Same

DECLARATION

Honorable Commissioner
Washington, D.C. 20231

Sir:

I, Masahiko MUTAI, of 988, Shimizu 4-chome, Higashiyamato-shi, Tokyo, Japan, hereby declare and state as follows:

1. I graduated from the Faculty of Veterinary Science, Obihiro Veterinary College, Obihiro City, Hokkaido, Japan, with the degree of Bachelor of Veterinary Science on October 1943. I further studied as an Assistant at Obihiro Veterinary College, Faculty of Veterinary Science from June 1944 and successively studied as an Assistant Professor at Obihiro Veterinary College from October 1945. From January 1950, I began to study both medical and biological courses as an Assistant at the Institute for Infectious Disease (now changed to the Institute of Medical Science), University of Tokyo, and continuously studied as an Assistant Professor at the same Institute from October 1962. During my study at the Institute for Infectious Disease, University of Tokyo, I received the degree of Ph.D. in Medicine on February 1959 with a theme of "Isolation of Measles Virus."

2. I was engaged as a leading research staff at Osaka Prefectural Institute of Public Health for the period from

March 1963 to March 1967 and then at Yakult Institute for Microbiological Research for the Period from April 1967 to February 1982 to study Medical Science, especially Microbiology and Virology. Since March 1982, I am now the Head of Yakult Central Institute for Microbiological Research, Tokyo, Japan, and take care of all kinds of researches made in the Institute.

3. Aside from the works engaged in Yakult Central Institute for Microbiological Research, I am a member of the following major Scientific Societies:

(1) The Japanese Biochemical Society

(2) Japanese Society for Bacteriology

(3) Society of Japanese Virologists

(4) The Japanese Cancer Association.

4. I have written alone or with co-workers a number of biochemical and medical articles and reported them in the relevant academic journals and magazines. Of these articles, the main ones are as listed below:

(1) M. Mutai, Isolation and Identification of Measles Virus, Jap. J. Exp. Med., 29, 283-295, (1959);

(2) H. Wako, M. Mutai et al., Clinical and antigenic effects in children of Measles Virus adapted to bovine kidney cell culture, Jap. J. Exp. Med., 31, 481-485,(1961);

(3) M. Matumoto, M. Mutai et al., Live Measles Virus Vaccine: Clinical trial of vaccine prepared from a variant of the Sugiyama strain adapted to bovine kidney cells, Jap. J. Exp. Med., 32, 433-448, (1962);

(4) M. Matumoto, M. Mutai et al., A neurotropic variant of Measles Virus in suckling mice, Arch. Gesamte Virusforschung, XIV, 683-696, (1964);

(5) S. Hashimoto, M. Mutai et al., Dimethylnitrosamine

formation in the gastro-intestinal tract of rats, Fd.Cosmet. Toxicol., 14, 553-556, (1976);

(6) Y. Umezaki, M. Mutai et al., Characterization of acetate uptake by the colonic epithelial cells of the rat, Oflugers Arch., 388, 205-209, (1980);

(7) Tohyama,K., Mutai,M. et al., Effect of Lactobacilli on Urinary Indican Excretion in Gnotobiotic Rats and in Man, Microbiol. Immunol: 25, 101-112, (1981);

(8) Yajima, T., Mutai, M. et al., Effect of Short-chain Fatty Acids on Electrical Activity of the Small Intestinal Mucosa of Rat, Life Sciences, 28, 983-989, (1981);

(9) M. Mutai et al., Factors influencing bacterial colonization, Recent Advances in Germfree Research, (Ed. by S. Sasaki et al.), 149-157, Tokai University Press, 1981;

(10) Kato, I., Mutai, M. et al., Antitumor Activity of Lactobacillus casei in Mice, Gann., 72, 517-523, (1981);

(11) Uchida, K., Mutai, M. et al., Spontaneous Pituitary Changes and their Influence on Mammary Glands in SD Female Rats, Exp. Anim., 30, 421-433, (1981);

(12) Tanaka, R., Mutai, M. et al., Effects of Administration of TOS and Bifidobacterium breve 4006 on the Human Fecal Flora, Bifidobacteria Microflora [2], 17-24, (1983);

(13) Kato, I., Mutai, M. et al., Macrophage Activation by Lactobacillus casei in Mice, Microbiol. Immunol. [27], 611-618, (1983); and

(14) H. Nagata, M. Mutai et al., Effect of an antitumor alkaloid, camptothecin, and its derivatives on cell growth of mammalian cells in culture, J. Aichi Med. Univ. Assoc., 11, 286-293, (1983).

5. I am one of the inventors of the invention in U.S. Patent Application Serial No. 627,980 (referred to hereinafter simply as "the present application" and the invention thereof simply as "the present invention"). As a matter of course, I am fully aware of the gist and construction of the present invention relating to new camptothecin derivatives and processes for preparing same as well as pharmacological usefulness of the new camptothecin derivatives for tumor therapy. Further, I am familiar with the contents of Sugasawa (Ref. C), Winterfeldt et al. (Ref. D), Miyasaka et al. (Ref. M) or Miyasaka et al. (Ref. O) which were published prior to the convention priority dates of the present application and thus cited thereto by the Examiner as prior arts.

6. With a view to demonstrating the significant distinction of the compounds of the present invention based on their unexpectedly strong anti-tumor activity and improved therapeutic indices from the known compounds of the cited references described above, I have made comparative experiments together with other supporting staffs to compare anti-tumor activities in mice under my supervision of camptothecin derivatives of the present invention with those of the reference compounds, 7-ethyl-10-hydroxy camptothecin disclosed in Ref. O.

I add that the comparative experiments per se have been carried out in cooperation of other assisting staffs.

7. I am fully assured of the following methods for the comparative experiments and the following results thereof.

Materials and Methods used for
the Comparative Experiments

Test Compounds

The following compounds were used in the test.

Compound No.	Name
1	7-Ethyl-10-[4-(isopropylcarbamoylmethyl)-1-piperazino]carbonyloxy camptothecin HCl-salt
2	7-Ethyl-10-(1-piperazino)carbonyloxy-camptothecin HCl-salt
3	7-Ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin HCl-salt
4	7-Ethyl-10-hydroxycamptothecin Na-salt (control)

Test Animals

Healthy BDF_1 female mice 7 weeks of age (22-23 g in body weight) were purchased from Charles River, Japan, Inc. for the comparative experiments. Throughout the experimental period, food and water were provided ad libitum and the animals were maintained in an air-conditioned animal room. Several groups of these mice were used for the comparative experiments, each group being composed of ten mice.

Tumor System

Mouse lymphatic leukemia L-1210 available from the National Cancer Center was used for the experiments.

Vehicle for Tumor System and Test Compounds

A sterilized physiological saline was used as the vehicle for inoculation of the tumor cells and for administration of the test compounds.

Inoculation of Tumor Cells

A physiological saline containing tumor cells of the mouse lymphatic leukemia L-1210 was prepared, in which the contents of the tumor cells had been adjusted so that $2\text{-}3 \times 10^5$ tumor cells might be contained in 0.2 ml of the solution, and inoculated intraperitoneally to each mouse.

Administration of the Test Compounds

The test compound was dissolved in the vehicle so that 0.1 ml of the resultant solution might contain a necessary daily dose of 0.3 - 80 mg per kg of body weight of the test animal, and administered once a day to a group of the test animals intraperitoneally one day after the inoculation of the tumor cells and for the following 4 days. On the other hand, the vehicle alone was given in the same manner as described above to another group of the test animals as control. The total dose of the test compounds administered to the test animals was 1.56 - 400 mg/kg.

Evaluation of Anti-tumor Activity

(1) After inoculation of the tumor cells, survival days of the treated group of the test animals (T) and survival days of the control group of the test animals (C) were recorded and a survival ratio, T/C (%), was calculated according to the following equation:

$$T/C\ (\%) = \frac{\text{mean survival days of the treated mice}}{\text{mean survival days of the control mice}} \times 100.$$

For a useful anti-tumor agent, it is of course necessary that the T/C ratio must be larger than 100 and the anti-tumor activity is evaluated as increasing as the T/C ratio increases above than 100, and usually above 120.

(2) The number of mice alive for 40 days after the inoculation of tumor cells was recorded for each of the test compounds.

(3) Drugs must be evaluated by their balancing their therapeutic effect toward disorders and their adverse effects, such as toxicity. This point is particularly important for anti-tumor drugs. Based on this understanding, a therapeutic index (TI)

of each test compound was calculated according to the following equation:

$$TI = \frac{\text{maximum tolerate dose}}{\text{minimum effective dose}} .$$

Results

The anti-tumor activities of the test compounds in terms of maximum T/C ratio are shown in Table 1 below. The number of mice alive for 40 days for the test compounds are also shown in Table 1.

Table 1

Compd. No.	Dose for the maximum T/C	T/C (%)	Number of mice alive for 40 days	TI
1	200 mg/kg	490	4/6	128(200/1.56)
2	100	424	3/6	128(200/1.56)
3	50	545	5/6	64(100/1.56)
4	150	190	0/6	50(150/3)

Discussion

The result of the above Comparative Experiments reveals that the compounds of the present invention (Compd. Nos. 1-3) are excellent in T/C and, especially, the number of mice alive for 40 days as compared with the compound of Ref. O (control, Compd. No. 4). Thus, the present invention is firmly believed to contribute greatly to the chemotherapy of cancer.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that

these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S. Code 1001 and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

Dated: November 20, 1985

Masahiko Mutai

Masahiko MUTAI